Detection of Intraspecific Diversity of
Heterodera glycines Using Isozyme Phenotypes[1]

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Abstract: Twelve populations of *Heterodera glycines* from the United States (8), China (2), Japan (1), and Colombia (1) were surveyed for phenotypic intraspecific variability in 42 enzyme systems. Activity of 20 enzymes was detected following isoelectric focusing in polyacrylamide gels of extracts from mass homogenates and single females. Five enzymes, aspartate aminotransferase, phosphoglucose isomerase, α- and β-esterases, and hexokinase were the most useful for detecting intraspecific variability. Phenotypic variability between single females was best demonstrated with α- and β-esterases and acid phosphatase enzyme systems. These results suggest that isoelectric focusing in conjunction with sensitive enzyme systems can be used to detect phenotypic variation between individual nematodes from the same population. The unusual phenotypic variability detected in the *H. glycines* population from Virginia indicates that the genetic diversity of this population is complex.
Key words: enzyme, genetic diversity, *Heterodera glycines*, intraspecific variability, isoelectric focusing, isozyme, race.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is one of the most extensively studied plant-parasitic nematodes. It has been found in 25 states in the central and southeastern United States. The principal control strategies are the use of resistant varieties and crop rotation. The extreme genetic variability in SCN populations makes the effective use of resistant varieties increasingly difficult (20,30). Evidence of resistance breaking types or variability in virulence in SCN populations has been reported in the United States and Japan (4,21,27,28). Riggs et al. (26) separated 30 populations of SCN into 25 races based on their ability to mature on 13 soybean genotypes.

Classical taxonomic studies of morphological features have proven ineffective in separating races or isolates of SCN. Koliopanos and Triantaphyllou (18) found considerable variation in tail length, tail terminus length, and body length of four SCN isolates but were unable to separate these isolates into distinct races based on host differentials. Likewise, Riggs et al. (25) analyzed 35 populations of *H. glycines* by morphometric and serological methods and subjected the data to BMDP (Biomedical Package) stepwise discriminate function analysis. They found no consistent grouping of these populations into races.

Early investigations using gel electrophoresis to analyze nematode proteins were successful in distinguishing various genera (5,9) and species (15–17,29). Dickson et al. (6) used soluble proteins and profiles of eight enzymes to separate *Meloidogyne hapla, M. incognita,* and *M. arenaria.* In an extensive study of 16 *Meloidogyne* species, Esbenshade and Triantaphyllou (8) obtained species-specific phenotype patterns for nonspecific esterases, malate dehydrogenase, superoxide dismutase, and glutamate oxaloacetate transaminase for *M. javanica, M. incognita* and *M. hapla.* The two sibling species, *Caenorhabditis elegans* and *C. briggsae* which are very similar morphologically and easily confused, were successfully distinguished by 22 of 24 enzyme systems (2).

Lawson et al. (19) used isoelectric focusing (IEF) to separate nematode egg proteins and distinguished four species of *Meloidogyne.* In a comparative electrophoretic analysis of soluble proteins extracted from *H. glycines* races 1 through 4, Pozdol and

TABLE 1. Geographic location, collection dates, and race designation of *Heterodera glycines* populations.

Location	Code	Date	Race†
North Carolina	NC	1982	2
Arkansas	AR-3	1959	3
Arkansas	AR-4B	1982	4
Arkansas	AR-B	1972	
Virginia	Va-6C	1970	3
Wisconsin	Wisc	1984	3
South Carolina	SC	1983	3
Arkansas	B-10	1984	4
Shandong Province, China	C20C	1981	5
Liaoning Province, China	C22B	1981	2
Japan	Jap	1982	
Colombia, South America	Col	1984	3

† Race tests according to Golden et al. (12).

Noel (22) used a modified discontinuous electrophoretic system and were unable to distinguish specific races. They were successful, however, in differentiating *H. glycines*, *H. schachtii*, *H. trifolii*, and *H. lespedezae*.

Most electrophoretic studies have used proteins obtained from mass homogenate preparations of many nematodes. In the last few years, gel electrophoresis has been used with single females in attempts to obtain quantitative genetic information (14,23). To observe genetic diversity in *H. glycines*, 12 populations from various geographic locations were surveyed electrophoretically. The purpose was to determine the similarities and differences among enzyme phenotypes and soluble proteins from both mass homogenates and single females by ultra-thin IEF.

MATERIALS AND METHODS

Nematode cultures: Populations of *H. glycines* were sent directly to R. D. Riggs or collected by the first author from different geographic regions. Races were determined on the standard soybean differentials (12) (Table 1). Nematodes were propagated on soybean (*Glycine max* (L.) Merr. cv. Essex) in the greenhouse at an ambient temperature of 20–28 C.

Protein extraction: For mass homogenates 25 white females, ca. 20 days-old, were placed in 1.5-ml Eppendorf tubes containing 7.0 µl extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 1 mM mercaptoethanol, pH 7.2). Nematodes were homogenized for 45 seconds at 4 C with an Eppendorf fitted teflon pestle (Kontes, Vineland, NJ) connected to a motor drive. The homogenates were centrifuged (15,600 *g*) in an Eppendorf microfuge for 2 minutes, and the supernatants were collected. The pellets were reextracted as described, and the two supernatants were pooled. The extracts were either used immediately or stored at −70 C until use.

Proteins were extracted from single females by hand picking and squashing individual females on 0.25-cm² pieces of filter paper (Whatman #1) using a stainless steel flat edge rod at 4 C. The filter paper pieces with crushed nematodes were placed directly on prefocused gels.

Electrophoresis: Proteins and isozymes from mass homogenates and single females were analyzed by analytical isoelectric focusing in thin layer (120–240 µm) polyacrylamide gels (24). The gel concentration consisted of T = 5% and C = 3%. Gel solutions (30 ml total) included 5.0 ml acrylamide (0.9 g/100 ml), 3.5 ml 87% glycerol, 1.5 ml 3–7 or 4–6 40% ampholytes (Isolab Inc., Akron, OH), and 15 ml deionized-distilled water. Gel solutions were degassed for 5 minutes, and 1.5 ml 10% ammonium persulfate (Sigma, St. Louis, MO) and 15 µl TEMED (Sigma, St. Louis, MO) were added to facilitate polymerization. Two gels (15.0 ml/gel) were cast with a syringe on Gel Bond polyester sheets (FMC Corp., Rockland, ME). Gel layers of 120 or 240 µm were cast with gaskets made from either one or two layers cut from Parafilm (Amercan Can, Greenwich, CT) sheets (24). Gels were allowed to polymerize for 1 hour and used the same day or stored overnight at 4 C.

An LKB Multiphor System was used with 0.5 M phosphoric acid as the anode buffer and 0.25 M sodium hydroxide as the cathode buffer. Gels were prefocused for 10 minutes at 10 watts (constant power), samples were applied, and the gels were run

TABLE 2. Enzymes examined and references to strains used in isoelectric focusing of *Heterodera glycines* extracts.

Enzyme	E.C. number†	Activity‡	Stain reference
Oxidoreductases			
Sorbitol dehydrogenase	1.1.1.14	+	Allendorf et al.
Shikimic acid dehydrogenase	1.1.1.25	+	Cheliak and Pitel
Glycerate-2-dehydrogenase	1.1.1.29	−	Cheliak and Pitel
Malate dehydrogenase	1.1.1.37	+	Allendorf et al.
Malic acid	1.1.1.40	+	Allendorf et al.
Isocitrate dehydrogenase	1.1.1.42	−	Allendorf et al.
Phosphogluconate dehydrogenase	1.1.1.44	+	Allendorf et al.
Glucose-6-phosphate dehydrogenase	1.1.1.49	+	Cheliak and Pitel
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	−	Harris and Hopkinson
Xanthine dehydrogenase	1.2.1.37	+	Allendorf et al.
Succinate dehydrogenase	1.3.99.1	+	Allendorf et al.
Glutamate dehydrogenase	1.4.1.3	−	Cheliak and Pitel
Diaphorase	1.6.4.3	−	Allendorf et al.
Nicotinamide adenine dinucleotide phosphate dehydrogenase	1.6.99.1	+	Cheliak and Pitel
Nicotinamide adenine dinucleotide dehydrogenase	1.6.99.3	+	Cheliak and Pitel
Cytochrome c oxidase	1.9.3.1	+	Cheliak and Pitel
Polyphenol oxidase	1.14.18.1	−	Cheliak and Pitel
Superoxide dismutase	1.15.1.1	+	Cheliak and Pitel
Transferases			
Aspartate aminotransferase	2.1.1.1	+	Allendorf et al.
Glutamate pyruvate transminase	2.6.1.2	−	Allendorf et al.
Hexokinase	2.7.1.1	+	Allendorf et al.
Phosphofructokinsae	2.7.1.11	−	Dunaway and Weber
Pyruvate kinase	2.7.1.40	−	Allendorf et al.
Nucleoside phosphotransferase	2.7.1.77	−	Allendorf et al.
Phosphoglycerate kinase	2.7.2.10	−	Allendorf et al.
Creatine kinase	2.7.3.2	−	Harris and Hopkinson
Adenylate kinase	2.7.4.3	−	Allendorf et al.
Phosphoglucomutase	2.7.5.1	+	Allendorf et al.
Hydrolases			
Esterases	3.1.1.1	+	Allendorf et al.
Cholinesterase	3.1.1.8	+	Harris and Hopkinson
Acid phosphatases	3.1.3.2	+	Harris and Hopkinson
Fructose biphosphatase	3.1.3.11	−	Allendorf et al.
β-glucosidase	3.2.1.21	+	Cheliak and Pitel
β-glucoronidase	3.2.1.31	−	Allendorf et al.
Peptidase	3.4.13.11	−	Harris and Hopkinson
Adenosine deaminase	3.5.4.4	−	Allendorf et al.
Lyases			
Phosphoenolpyruvate carboxylase	4.1.1.31	−	Cheliak and Pitel
Aldolase	4.1.2.13	−	Harris and Hopkinson
Fumarase	4.2.1.2	−	Allendorf et al.
Isomerases			
Triose phosphate isomerase	5.3.1.1	−	Allendorf et al.
Mannose 6-phosphate isomerase	5.3.1.8	−	Cheliak and Pitel
Phosphoglucose isomerase	5.3.1.9	+	Allendorf et al.

† Enzyme commission number. Enzyme Committee, International Union of Biochemistry Classification.
‡ + = activity detected; − = no activity detected.

for approximately 1 hour (final voltage 1,500–1,800 volts). The pH of the gels stained for soluble proteins was determined with pI standards 3–10 (Pharmacia Co., Piscataway, NJ) and for isozymes with a surface electrode (LKB Inc.). All enzyme stain recipes used, except phosphofructokinase (7), are described by Allendorf et

al. (1), Cheliak and Pitel (3), and Harris and Hopkinson (13). A summary of the enzymes examined and their detectability are listed in Table 2. Scanning densitometer analyses of gels were made on a Bio-Rad 1650 scanning densitometer. Coefficients of genetic similarities (Sm) were calculated from protein banding patterns (10).

$$Sm = \frac{\text{Number of bands of common mobility}}{\text{Maximum number of bands in an individual}}$$

RESULTS

Activities of 20 of the 42 enzyme systems investigated were detected by IEF of protein extracts from 12 populations of *H. glycines*; however, 12 of the 20 enzymes did not resolve well enough to permit precise comparisons.

Mass homogenates: Twelve *H. glycines* populations were placed into groups based on similarities of electromorphs (electromorph defined as a single resolved band) for each enzyme system tested. The α-esterase system contained three distinct phenotypic groups (Figs. 1, 3): 1) NC, AR-3, AR-4B, and AR-B populations; 2) Wisc, SC, B-10, C20C, C22B, Japan, and Col populations; and 3) Va-6C population. The Va-6C population was the most variable, exhibiting two electromorphs at pH 4.8 and pH 5.1. These two bands were not detected in the other 11 populations and were used as enzymatic markers for identifying this population.

The β-esterase exhibited similar phenotypes to the α-esterase with one exception. The B-10 population phenotype lacked a single electromorph at pH 5.7 (Fig. 2). The absence of this electromorph makes this population similar to the group containing the NC and three AR populations (AR-3, AR-4B, and AR-B). This was not the only population in which we detected differences between the two esterase systems.

Evaluation of the hexokinase system sep-

arated the 12 populations into two distinct phenotypic groups (Fig. 4). Eight populations (Va-6C, Wisc, SC, B-10, C20C, C22B, and Col) had one electromorph, whereas the NC and three Arkansas populations (AR-3, AR-4B, and AR-B) showed two identical migrating electromorphs (Fig. 4).

Three different phenotypes were resolved in the phosphoglucose isomerase system (Figs. 5, 7). The NC, AR-3, AR-4B, and AR-B populations each had a single electromorph at pH 4.6. The Va-6C contained the most unusual phenotype because of the absence of one electromorph at the pH 4.6 locus. The remaining seven populations each had two electromorphs at pH 4.6 and 4.4.

Staining for aspartate aminotransferase also proved valuable in detecting differences among populations. The 12 populations were separated into three phenotypic groups (Fig. 6). The predominant phenotype was exhibited by the NC, AR-4B, AR-B, C20C, and Japan populations. The second major phenotype included the AR-3, Wisc, B-10, and Col populations. A unique phenotype not detected in any other population was resolved in the Va-6C population. This phenotype was characterized by a slow migrating electromorph at pH 4.8.

Other enzyme systems that exhibited high levels of phenotypic homogeneity among the 12 populations were nicotinamide adenine dinucleotide phosphate dehydrogenase (Fig. 8), cholinesterase (except Va-6C) (Fig. 9), nicotinamide adenine dinucleotide dehydrogenase (Fig. 10), shikimic acid dehydrogenase, superoxide dismutase, and β-glucosidase (data not shown).

Isoelectric focusing gels stained for soluble proteins showed extensive homology. Such a gel, with its corresponding densitometer scans, is shown in Figures 11 and 12. We did not detect major differences in electromorphs among populations of *H. glycines*; however, subtle differences in protein concentrations were detected using scanning densiometry. Similarity coefficients among the 12 populations of *H. glycines* were generated from protein profiles
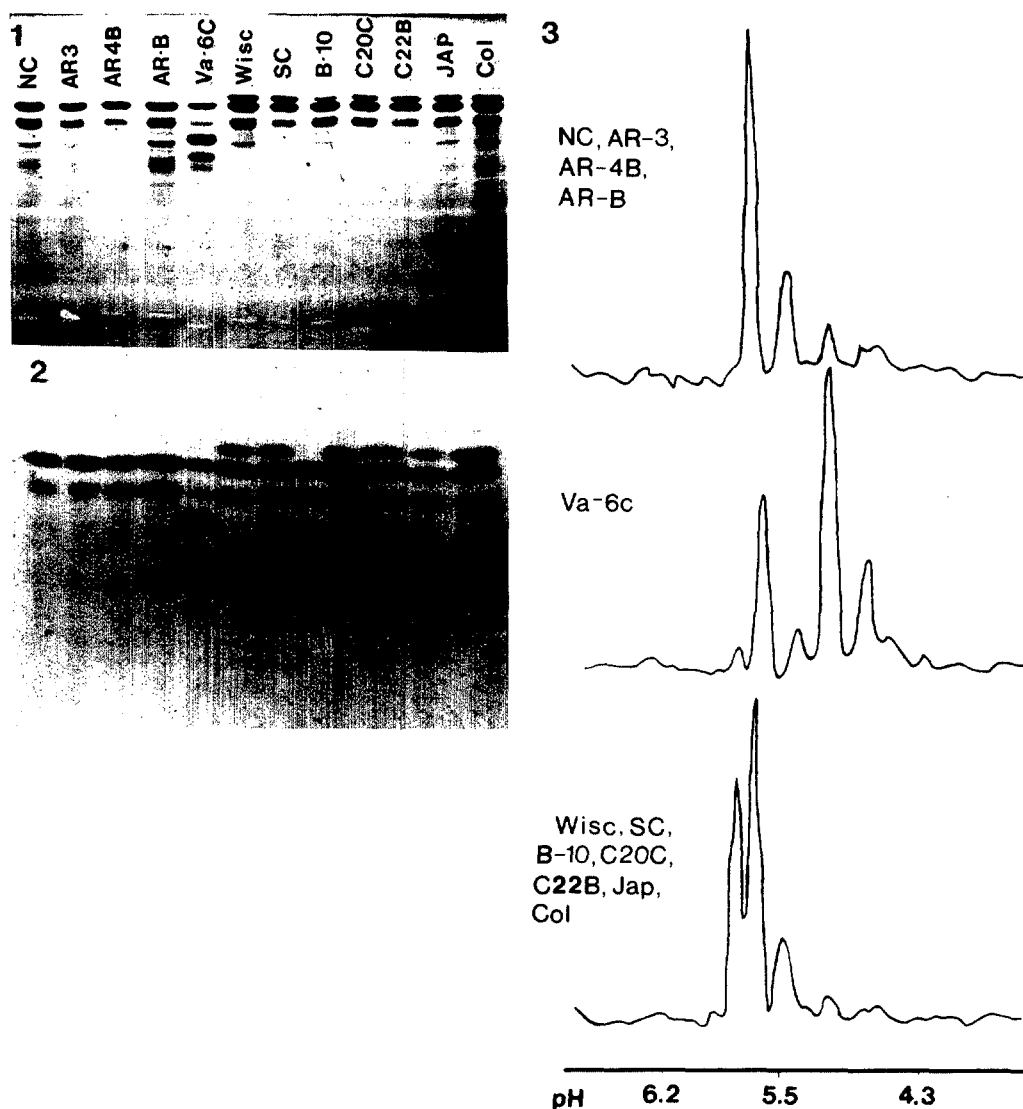
FIGS. 1–3.   Isoelectric focusing gels (pH 4–6) of esterase isozymes from *Heterodera glycines* populations. 1) α-esterase (α-naphthyl acetate substrate). 2) β-esterase (β-naphthyl acetate substrate). Lanes are in the same order as in the first figure. Arrow indicates the missing electromorph. 3) Densitometer scans of α-esterase gel indicating three phenotypic groups.

stained with Coomassie blue and read through a scanning densitometer (Table 3).

Single nematode analyses: Acid phosphatase staining identified 4–8 electromorphs (Fig. 13). Individuals from the same populations exhibited similar banding patterns. Interpopulation variability was detected between specific populations; for example, individuals from the C20C population did not contain an electromorph found in most other populations.

The nonspecific esterases exhibited significant levels of variability in 9 of the 12 populations tested (Figs. 14, 15). All individuals from the 12 populations contained two homogeneous electromorphs located at pH 5.3 and pH 5.5. The other electromorphs for this enzyme were polymorphic. Individuals from three populations, B-10,
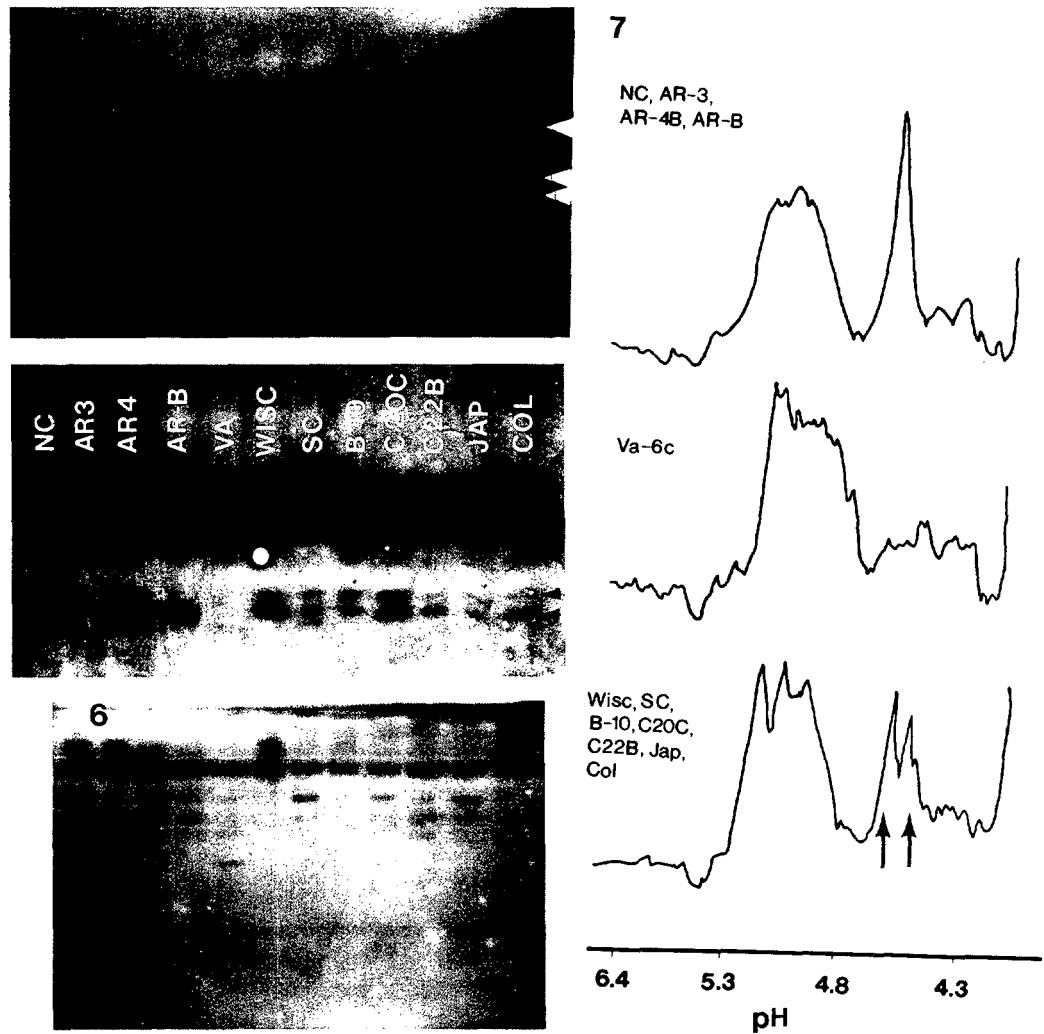
FIGS. 4–7. Isozyme phenotypes of *Heterodera glycines* populations. Lanes are in the same order for the three figures. 4) Hexokinase, indicating two phenotypic groups. 5) Phosphoglucose isomerase, indicating three phenotypic groups. 6) Aspartate aminotransferase exhibiting polymorphic phenotypes. 7) Densitometer scans of phosphoglucose isomerase.

TABLE 3. Coefficients of similarity among 12 populations of *Heterodera glycines* based on IEF of soluble proteins.

	NC	AR-3	AR-4B	AR-B	Va-6C	Wisc	SC	B-10	C20C	C22B	Jap	Col
NC		0.94	0.86	0.84	0.82	0.91	0.93	0.94	0.98	0.93	0.97	0.94
AR-3			0.99	0.90	0.78	0.99	0.90	0.88	0.87	0.88	0.91	0.89
AR-4B				0.87	0.86	0.95	0.86	0.86	0.92	0.84	0.86	0.89
AR-B					0.83	0.89	0.92	0.93	0.87	0.87	0.91	0.88
Va-6C						0.83	0.78	0.82	0.83	0.84	0.79	0.82
Wisc							0.96	0.85	0.92	0.94	0.86	0.90
SC								0.89	0.93	0.86	0.94	0.88
B-10									0.92	0.90	0.92	0.90
C20C										0.92	0.88	0.93
C22B											0.97	0.94
Jap												0.86

Source: Ferguson (10).
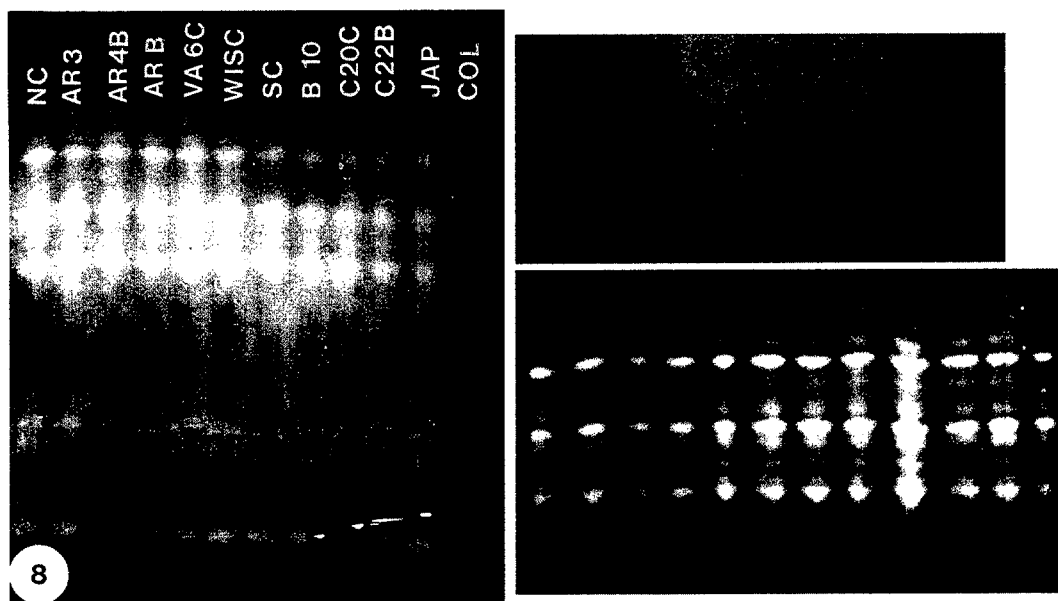
FIGS. 8–10.   Isoelectric focusing gels (pH 4–6) showing homogeneity among *Heterodera glycines* populations. Lanes are in the same order for the three figures. 8) Nicotinamide adenine dinucleotide phosphate dehydrogenase. 9) Cholinesterase (α-naphthyl acetate substrate, TR red salt). 10) Nicotinamide adenine dinucleotide dehydrogenase.

AR-3, and AR-B, were homogeneous for two electromorphs and exhibited identical phenotypes. The Va-6C population had the most variable phenotype for α-esterase. Individuals in this population had two and sometimes three electromorphs (pH 4.9 and pH 5.1) that were consistently found only in individuals from this population.

The frequency of some esterase electromorphs ranged from 10% in the NC population to as high as 50% in the SC population. These frequencies of electro-
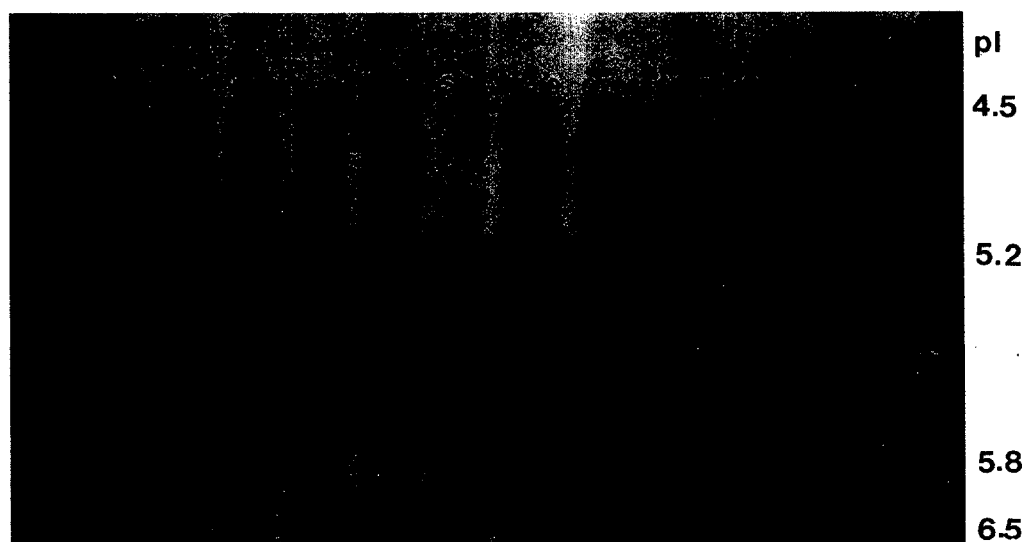

FIG. 11.   Isoelectric focusing (pH 4–6) of soluble proteins from *Heterodera glycines* stained with Coomassie brilliant blue R. pI standards are in the far right lane.
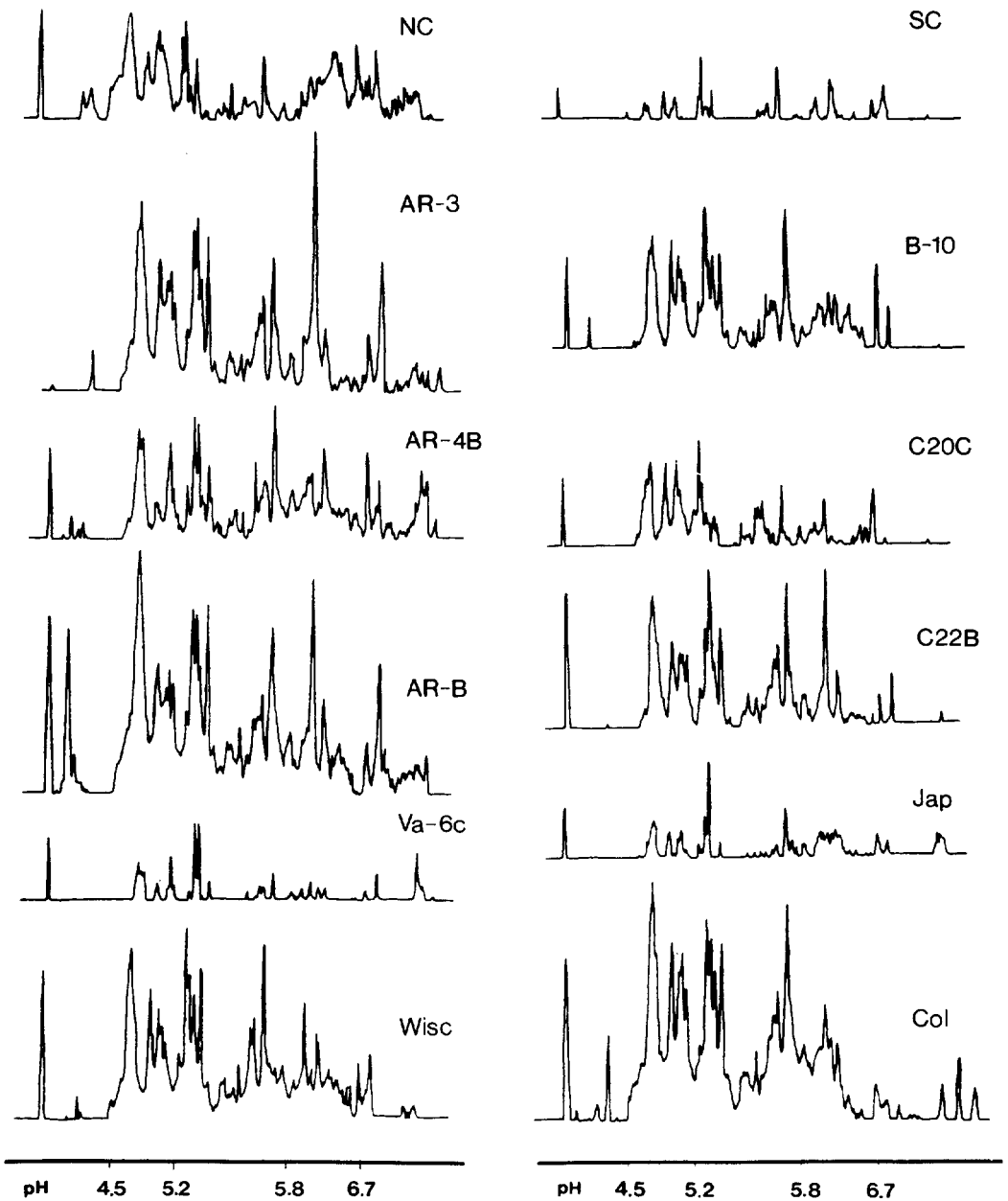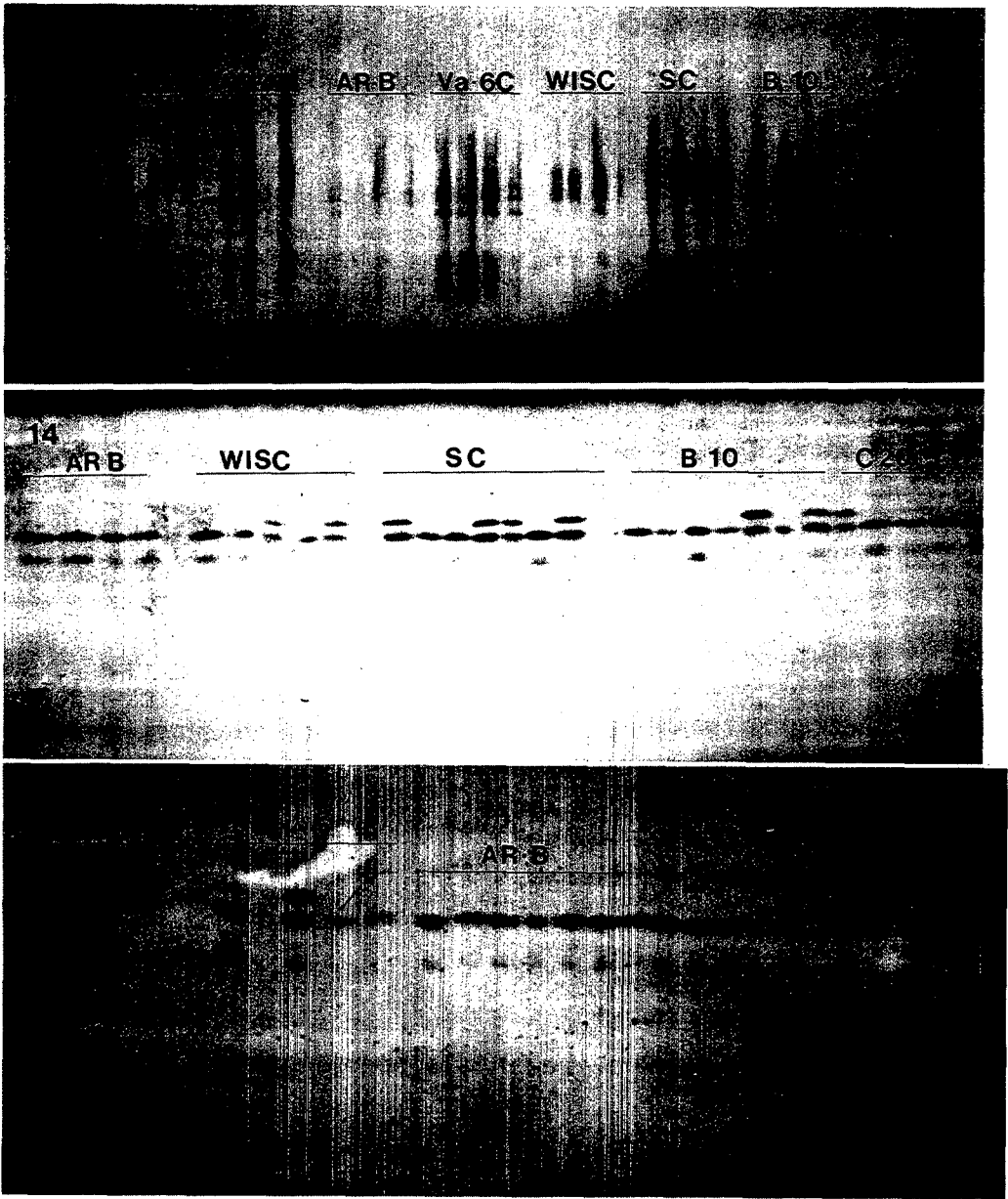
12

FIG. 12.   Densitometer scans of isoelectric focusing (pH 4–6) gel of soluble proteins from *Heterodera glycines* populations.

morphs varied over time but were never detected in populations exhibiting homogeneous electromorphs (B-10, AR-3, and AR-B).

DISCUSSION

The initial intent of this work was to quantify genetic differences (allelic fre-

Figs. 13–15. Isozyme phenotypes of single female *Heterodera glycines* from selected populations. 13) Acid phosphatase. Arrows indicate sites of polymorphic electromorphs. 14) α-esterase, indicating monomorphic and polymorphic electromorphs. 15) β-esterase.

quencies, heterozygosity values) from diverse geographic populations of *H. glycines* using single individuals in conjunction with at least 20 enzyme systems. The amount of extractable protein from a single cyst nematode, however, was insufficient for detection of the enzymes. Because of these problems, analysis of mass homogenates was necessary and practical.

Polyacrylamide isoelectric focusing of mass homogenates and subsequent enzyme staining were successful in detecting phenotypic variation among certain populations of *H. glycines* from North America,

China, Japan, and South America. Only 8 of 42 enzymes tested could be resolved efficiently.

The α- and β-esterases and acid phosphatase systems showed that distinct levels of genetic variability exist among individuals in populations originating from various geographic locations. Interpretating and resolving multiple locus enzymes using IEF is exceedingly difficult. The increased sensitivity of IEF over starch gel electrophoresis can result in resolution of age specific isozymes when different stages of individuals are used in protein homogenates.

Analyses of mass homogenates of 12 populations using the eight enzyme systems showed some consistent grouping among populations. The NC, AR-3, AR-4B, and AR-B populations had identical phenotypes for α-esterase, β-esterase, hexokinase, and phosphoglucose isomerase. In no case did isozyme analyses of mass or individual homogenates from populations correlate with the conventional race scheme based on quantitative reproduction on a set of soybean differentials. At the onset of this project, the NC, AR-3, AR-4B, and AR-B populations corresponded to races 2, 3, 4, and Bedford, respectively, but the parasitic capabilities varied in a nonpredictable manner during the course of this investigation.

The Va-6C population deserves further investigation. This population exhibited unique and variable phenotypic profiles unlike the other populations of *H. glycines* examined. This aberrant population has a life cycle of approximately 18 days, compared with 24–26 days for the other populations, and displays low similarity coefficients in the range comparative to sibling species.

Before this study other techniques had been used to investigate genetic variability among *H. glycines* populations. Ferris et al. (11) analyzed isolates of *H. glycines* by two-dimensional gel electrophoresis. Two of their isolates were classified as race 3 populations by standard soybean differentials. These isolates gave common protein patterns but exhibited a number of quanti-

tative differences, and the authors concluded that the current race concept for *H. glycines* obscures extensive differences among populations. On the basis of our results, we agree with their conclusion. Presently, we believe that integration of electrophoretic techniques into the existing race classification system would be difficult. Electrophoretic techniques can be used, however, to detect precisely interspecific and infraspecific genetic diversity in nematodes. In addition, these techniques can be used for identification of genetic markers in hybridization experiments and for species verification.

Isoelectric focusing in conjunction with certain enzyme-staining systems was successful in detecting intraspecific variability between populations of *H. glycines* and was sensitive enough to detect polymorphisms within populations. Possible improvements of this technique, such as the addition of protein protectants or antioxidants or the incorporation of substrates directly into the gels to improve enzyme sensitivity, might be of additional value. Despite some current shortcomings, isoelectric focusing provides a useful tool in studying protein polymorphism and genetic diversity within and between nematode populations.

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